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Environmental Chemicals and Fetal Testis Development of Human and Rat

ANA ISABEL ALVES GIL BARRERA CALARRÃO

DISSERTAÇÃO

MESTRADO EM BIOLOGIA HUMANA E AMBIENTE

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Dissertação orientada por:

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Para ti,
Que do meu lado estiveste
E estarás,
Sempre.

Se depois de eu morrer, quiserem escrever a minha biografia,
Não há nada mais simples
Tem só duas datas — a da minha nascença e a da minha morte.
Entre uma e outra cousa todos os dias são meus.

Alberto Caeiro
"Se Depois de Eu Morrer, Quiserem Escrever a Minha Biografia"

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Thanks to all my friends and family, for always supporting and encouraging me. Thanks to Nana, for patience and guidance. To Mum and Dad, I could write a whole thesis with all the things that I thank you – to make a long story short, thank you for everything, you are the best.

Thanks to my love, for being always there for me and never hesitating when rough decisions arise; for fighting and struggling by my side for a better life; for being the best there is.

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List of Abbreviations

AA	Arachidonic Acid
ANOVA	One-way analysis of variance
ChaGP	Chicken anti-Goat Peroxidase secondary antibody
ChaMP	Chicken anti-Mouse Peroxidase secondary antibody
CIS	Carcinoma-*in-situ*
COX	Cyclooxygenase
DAB	3,3-diaminobenzidine tetrahydrochloride
DAPI	4',6-diamidino-2-phenylindole
DEHP	Diethylhexyl phthalate
DBP	Di(*n*-butyl) phathalate
e	Embryonic day
H_2O_2	Hydrogen peroxidase
Ki67	Ki67 Antigen
MAGE-A4	Homo sapiens melanoma antigen family A, transcript 4
MBP	Monobutyl pthalate
MEHP	Mono-(2-ethylhexyl) phthalate
MPW	Masculinization programming window
NChS/TBS/BSA	Normal chicken serum diluted 1:5 in TBS containing 5% (w/v) bovine serum albumin
NGS/TBS/BSA	Normal goat serum diluted 1:5 in TBS containing 5% (w/v) bovine serum albumin
NSAID	Nonsteroidal anti-inflammatory drugs
OCT4/3	Octamer binding transcription factor 3/4
pnd	Postnatal day
SRY	Sex-determining region Y
Streptavidin-HRP	Streptavidin labelled horseradish peroxidase enzyme conjugate
TBS	Tris-buffered saline; 0,05M Tris and 0,85% NaCl; pH 7,6
TBST	TBS + 0,05% Tween
TDS	Testicular Dysgenesis Syndrome
TGCC	Testicular Germ Cell Cancer
Tris	Tris(hydroxymethyl)aminomethane; $(HOCH_2)_3CNH_2$
Tyramide-Cy3 / Cy5 / Fl	Tyramide Signal Amplification^{TM} – Plus Cyanine3 / Cyanine5 / Fluorescein

Portuguese Abstract

Ao longo das últimas cinco décadas tem vindo a registar-se um decréscimo da taxa de fecundidade humana. Embora existam vários factores que contribuam para tal (por exemplo, alterações nos comportamentos sociais), são cada vez mais comuns problemas fisiológicos masculinos, como a diminuição da concentração e qualidade espermática, que devem ser também considerados importantes. Adicionalmente, doenças como hipospádias, criptorquidismo e cancro testicular têm também vindo a aumentar de incidência. Devido ao curto período de tempo em que se registam estas grandes diferenças, parece pouco provável que haja apenas influência genética a actuar. Como em tantos outros problemas recentes, a combinação das variações do estilo de vida e da exposição ambiental a moléculas perigosas pode prejudicar o desenvolvimento do sistema reprodutor masculino, diminuindo assim a fertilidade. Foi proposta uma origem comum na vida fetal para as doenças acima referidas, em que uma função deficiente das células somáticas do testículo fetal resultaria numa ruptura da organização e desenvolvimento normal, traduzindo-se em problemas reprodutivos masculinos. Esta teoria dá pelo nome de Síndrome da Disgénese Testicular (Testicular Dysgenesis Syndrome, TDS) e mostra o quão importante é o desenvolvimento fetal das estruturas reprodutivas masculinas para uma saúde e função reprodutiva normal posterior. Uma das doenças incluídas nesta TDS é o cancro testicular com origem na linha germinal (testicular germ cell cancer, TGCC), ao qual é atribuído a maioria das mortes relacionadas com problemas de saúde em homens entre os 15 e os 35 anos e cuja incidência duplicou nos últimos 40 anos. Durante o desenvolvimento fetal no Homem, as células germinais masculinas indiferenciadas mantêm a sua proliferação em níveis baixos após a perda de pluripotência e passagem para um estado diferenciado. Esta perda de pluripotência parece conter a hipótese de como a TGCC surge nos humanos, visto que se o processo for perturbado por factores externos, as células podem entrar num estado intermediário em que mantêm a taxa de proliferação alta, adquirindo características cancerígenas. Um tipo de molécula ambiental ao qual os fetos estão expostos, e que já demonstrou afectar negativamente o sistema reprodutor masculino noutras espécies, são os ftalatos, componentes industriais cuja exposição em roedores levou ao aparecimento de condições semelhantes às da TDS. No entanto, não há informações sobre o seu efeito nos humanos. Outros químicos ambientais cujo efeito no desenvolvimento reprodutivo pode ser negativo são os anti-inflamatórios não esteróides, tais como o paracetamol e a indometacina. Embora o mecanismo exacto ainda não seja conhecido, ambos actuam através da inibição da produção ou acção de prostaglandinas, que são lípidos libertados pelas células quando ocorre uma inflamação, embora a indometacina seja considerada um supressor mais inequívoco do que o paracetamol. A administração de paracetamol e outros analgésicos demostrou ter efeitos negativos na saúde reprodutiva em roedores. Esta tese centrou-se no estudo do efeito da exposição a

químicos ambientais como o ftalato di(*n*-butyl) (di(*n*-butyl) phthalate, DBP), paracetamol e indometacina no desenvolvimento e diferenciação das células germinais fetais, baseando-se em estudos preliminares e não publicados desenvolvidos pelo grupo de investigação em que me inseri. Para estudar o efeito do DBP, foram recolhidos testículos de fetos humanos (n = 8), com idades compreendidas entre as 14 e 20 semanas de gestação, para serem xeno-enxertados debaixo da pele dorsal de ratos imunodeficientes adultos (machos castrados) em porções pequenas. Sete dias após esta operação, os quais permitem o estabelecimento de irrigação sanguínea nos enxertos, os hospedeiros recebem doses diárias de controlo, DBP ou MBP (ftalato monobutyl, o metabolito activo do DBP) dissolvidos em óleo (500mg/kg/dia), durante 21 dias. Os hospedeiros ainda são sujeitos a três injecções semanais de gonadotropina coriónica humana (20 IU), para replicar as condições normais de gravidez e manter a produção de testosterona. Após o período de tratamento, os enxertos são recolhidos, fixados, cortados em secções e sujeitos a uma imunofluorescência tripla. No total, analisaram-se pelo menos dois enxertos controlos e dois expostos a DBP/MBP para cada feto, resultando, no mínimo, em 32 amostras. O protocolo de imunofluorescência permite visualizar células germinais com antigénios para OCT3/4 (células germinais indiferenciadas), MAGE-A4 (células germinais diferenciadas) e Ki67 (células em proliferação). As imagens foram obtidas por microscopia confocal e as células incluídas num túbulo seminífero definido, que demonstrassem fluorescência para estes antigénios, foram assumidas como pertencendo à linha germinal e contadas manualmente. Os valores registados para os enxertos-controlo e expostos a DBP, do mesmo feto, foram condensados até se obter um valor médio respectivo. A média dos enxertos controlo e dos expostos ao tratamento, de cada um dos fetos, foi utilizada então para análise estatística, usando-se um Teste *t* para amostras emparelhadas (significância: $P<0.05$). Os resultados mostraram que os enxertos expostos ao DBP(MBP) registam uma diminuição significativa de mais de 10% na percentagem de células germinais positivas para OCT3/4 (indiferenciadas). Por outro lado, a percentagem de células germinais positivas para MAGE-A4 (diferenciada) em enxertos tratados com DBP(MBP) foi significativamente maior do que os controlos. Porém, a proliferação das células germinais indiferenciadas e diferenciadas não registou alterações significativas em comparação com as amostras controlo. As células germinais reduziram a expressão de OCT3/4 para aumentarem a expressão de MAGE-A4 quando se diferenciaram, o que coincidiu com a redução, e eventualmente a perda, da capacidade proliferativa, demonstrando que o modelo dos xeno-enxertos consegue recapitular o desenvolvimento fetal normal das células germinais masculinas nos humanos. Estes resultados parecem dever-se a uma apoptose selectiva das células indiferenciadas. Se a apoptose fosse geral, a proporção de células germinais indiferenciadas e diferenciadas não mudava após o tratamento com o DBP(MBP), o que não está de acordo com os resultados obtidos, já que há um aumento de células germinais diferenciadas. Devido à falta de dados sobre o efeito do DBP nas gónadas

masculinas humanadas, esta hipótese estaria de acordo com dados anteriores de estudos *in vitro* e *in vivo*, em humanos e roedores respectivamente, em que a exposição a DBP reduz o número de células germinais fetais positivas para OCT3/4.

O segundo projecto desenvolvido e abordado nesta tese foi analisar os efeitos da exposição ao paracetamol e indometacina no número de células germinais nos testículos fetais de ratos. Fêmeas prenhas de ratos Wistar foram tratadas diariamente com paracetamol (350mg/kg/dia), indometacina (1mg/kg/dia ou 0.8mg/kg/dia) ou tratamento controlo. O tratamento decorreu entre o dia embrionário (e) 15.5 até ao e20.5, sendo os testículos dos descendestes recolhidos ao e21.5. As amostras foram fixadas, seccionadas e processadas através de um protocolo imunohistoquímico para o antigénio VASA (marcador típico de células germinais). O número de células germinais foi obtido por estereologia, que é um método uniforme e sistemático que permite analisar a composição celular de um tecido tridimensional, neste caso o testículo, através da contagem de núcleos de células germinais em secções bidimensionais desse mesmo tecido. Para as amostras fetais e21.5, foram contadas todas as células que expressassem VASA e que estivessem contidas num túbulo seminífero definido. A comparação entre amostras controlo e as expostas a paracetamol/indometacina foi analisada estatisticamente através de uma análise de variância ANOVA (significância: $p<0.05$). Os resultados mostraram uma redução significativa do número de células germinais em amostras expostas à indometacina, quando comparadas com as de controlo. Embora as gónadas masculinas expostas ao paracetamol não tenham apresentado uma diferença significativa, existe uma tendência para a redução do número de células germinais. O desenvolvimento normal das células germinais no rato passa por perderem totalmente a capacidade proliferativa quando se diferenciam, num processo síncrono e uniforme, ao contrário dos humanos. Não se sabe se o decréscimo do número de células pode ser atribuído a apoptose devido à falta de dados nesta área, mas a indometacina pode induzir um aumento da taxa de diferenciação das células germinais fetais, levando à paragem da divisão celular numa idade anormalmente precoce.

Outro ensaio experimental foi desenvolvido para investigar o efeito da exposição *in utero* à indometacina no número de células germinais em testículos de ratos obtidos no dia 25 após o nascimento (postnatal day, pnd25). O protocolo para investigar este efeito foi em tudo idêntico ao seguido para os testículos e21.5, excepto que não houve amostras expostas ao parecetamol e os testículos foram recolhidos após o nascimento (embora o período de exposição à indometacina tenha sido de e15.5 a e18.5). Visto que a gónada pnd25 já produz espermatozóides, as células germinais foram divididas em categorias que correspondem à progressão temporal da espermatogénese: espermatogónia, espermatócito I e espermatócito II. O número de células germinais de cada categoria, mais o número total de células germinais, foi contado também através de esterologia e a comparação estatística entre amostras controlo e tratadas com

indometacina foi feita com um Teste *t* de Student (significância: $p<0.05$). Os resultados mostraram que o número de espermatogónias e o número total de células germinais aumentaram significativamente em amostras tratadas com indometacina, enquanto os valores para espermatócitos I e II não sofreram alterações significativas. Isto sugere que a exposição *in utero* à indometacina possa induzir apoptose nas células germinais e aquelas que resistem conseguem recuperar, embora faseadamente, os seus números normais após o nascimento e cessação do período de tratamento. Além disso, ambos os ensaios em ratos mostram que, pelo menos em mamíferos, o desenvolvimento fetal normal das células germinais, e posterior fertilidade dos indivíduos, é dependente de prostaglandinas.

Este conjunto de estudos mostrou que a exposição fetal a químicos ambientais comuns, tais como os ftalatos e inibidores de prostaglandinas, pode afectar o desenvolvimento fetal das células germinais nos humanos e no rato, obtendo-se dados sobre este tema pela primeira vez.

English Abstract

Lifestyle and environmental exposure to hazardous molecules have been indicated as a cause for the decrease in human fertility. An increased incidence of hypospadias, cryptorchidism and testicular germ cell cancer has been reported and it has been hypothesized that these may form a Testicular Dysgenesis Syndrome (TDS) with a common origin in fetal life. Although testicular germ cell cancer (TGCC) occurs in young men, it appears that it arises from a failure of fetal germ cells to lose their pluripotency and which then transform into carcinoma-in-situ (CIS) cells, which are germ cells (GC) with pluripotency and proliferative features that probably lead to TGCC in young adulthood. It has been hypothesized that exposure in utero to some environmental factors could play a role in disturbing the testicular endocrine function in the fetus, possibly leading to TGCC. Di(n-butyl) phthalate (DBP), a common environmental chemical, has been shown to affect fetal testis development and endocrine function in the rat. Nonsteroidal anti-inflammatory drugs (paracetamol, indomethacin), which are believed to work through inhibition of prostaglandins, and other inflammation modulators can also affect fetal testis endocrine function in animal models. This study therefore aimed to assess the effect of DBP, paracetamol and indomethacin exposure on fetal GC development and differentiation. To study DBP effects, second-trimester human testis pieces from eight fetuses were xenografted under the backskin of nude castrated male mice, which were then treated with vehicle (control) or DBP for 21 days. Immunofluorescence for OCT3/4 (undifferentiated GC marker), MAGE-A4 (differentiated GC marker) and Ki67 (proliferative cell marker) was used to analyse GC differentiation and proliferation. Exposure to DBP led to a reduction in proportion of undifferentiated GC, although proliferative features were maintained, possibly due to selective apoptosis. To analyse the effect of indomethacin on GC numbers, e21.5 and pnd25 rat testes were evaluated in males exposed in utero to vehicle (control) or indomethacin (1mg/kg or 0.8mg/kg) treatment. Immunohistochemistry for VASA (GC marker) and stereology was used to assess GC number in e21.5 testes and spermatogenic temporal progression in pnd25 testes. Indomethacin exposed e21.5 rat testes showed a significant decrease in GC number, which is in agreement with preliminary results. Pnd25 rat testes from males exposed in utero to indomethacin showed an increase in spermatogonial numbers and total GC number, although the numbers for early and pachytene spermatocytes were not different. This shows that indomethacin affects germ cell development during the exposure period and that there might be a staggered recovery after birth toward normal GC number. Overall, these results show for the first time that exposure to common environmental chemicals affect human and rat male fetal GC development.

Introduction

Around 1.2 billion years ago, primitive eukaryotes started to reproduce sexually, which involves the recombination of parental genomes so that a new genotype is created. This allowed the emergence of more dynamic, adaptable life forms in a shorter period of time. So, sex and fertility are key factors to determine the future of our descendants. Being able to maintain fertility rates above levels of sustained population guarantees the existence of a given population as it is and reduces the risk of extinction, if no other external factors interact (such as a flood, for example).

The human species has suffered from variable fertility rates over its history. Nowadays, it is accepted that 2.1 is the minimum fertility rate to guarantee the sustainability of our species. Until two decades ago, some developing countries could reach a fertility rate of 8, and a general decrease has been noticed since. Postponing marriage and first births, associated with more women pursuing careers, are usually the most accepted reasons for the decrease in fertility rates, which in most Western countries do not even reach the replacement level (Skakkebæk *et al.*, 2006; Figure 1).

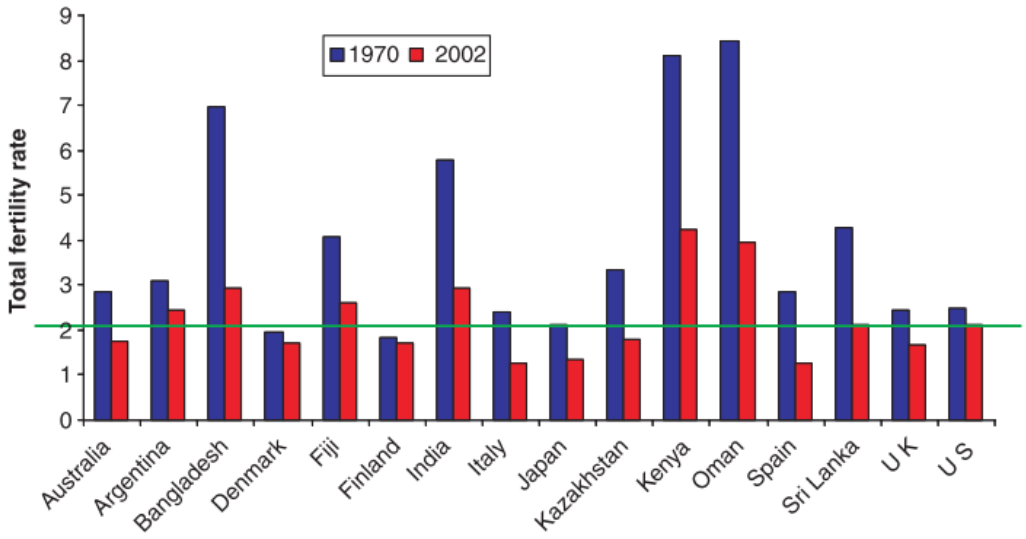
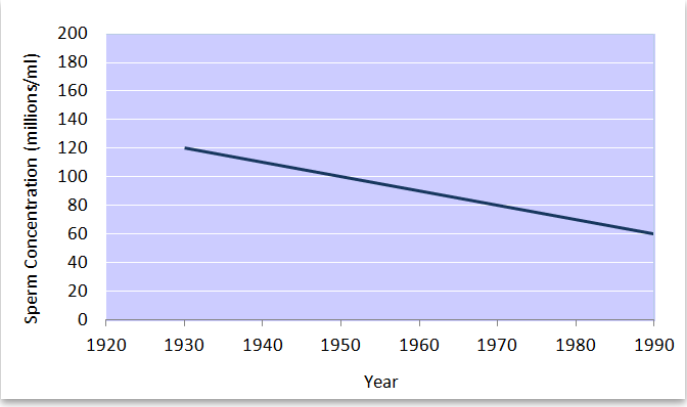


Figure 1. Fertility rates in 1970 (blue columns) and 2002 (red columns). The minimum fertility rate of 2.1 is also portrayed (green line) [Reproduced from Skakkebæk *et al.*, 2006].

Although social behaviour can contribute to this change in fertility rates, there are physiological changes that should also be considered, such as falling sperm counts (Figure 2) and other reduced sperm quality features (less motility and/or abnormal shape). Plus, the incidence of hypospadias, cryptorchidism and testicular cancer also seem to be increasing (Giwercman *et al.*, 1993; Skakkebæk *et al.*, 2006). With these great differences in a period of 50-70 years, it seems unlikely that only genetics plays an important role. Instead, changing lifestyle and environmental exposures to hazardous molecules over the years could impair the development of the male reproductive tract and result in a decreased fertility (Skakkebæk *et al.*, 2006).

Figure 2. Sperm counts declined in the last 70 years. Sperm counts have changed temporally, declining to nearly half in a 70-year period, as shown in a review of 101 studies [adapted from Swan *et al.*, 2000].



Testicular Dysgenesis Syndrome

Although spermatogenesis only starts after puberty and continues for the rest of life, the set-up for it is defined during fetal development (Sharpe, 2010). In humans, the primordial germ cell lineage can be traced back to the 4.5 day-old blastocyst, although it is not fully understood how they develop. It is formed outside of the presumptive gonad, which develops from the coelomic epithelium of the urogenital ridge, and can be found at the yolk sac, by the fourth week of gestation. The germ cells migrate through the gut into the mesoderm and arrive at the genital ridges, where their motile properties are lost. The undifferentiated gonad is then composed of germ cells and supporting coelomic cells (that give rise to Sertoli cells and Leydig cells) as well as other types of interstitial cells and Peritubular Myoid cells (Knobil & Neill, 1994). During the germ cell migration, the Sertoli cells begin to differentiate and aggregate around the germ cells to form spermatogenic cords, a critical process that is independent of testicular hormone production (George & Wilson, 1994; Brennan & Capel, 2004; Sharpe, 2010). After this, the final step is a new wave of cells from the

coelomic epithelium which differentiates into Leydig cells. These migrate into the interstitial regions and then begin to produce hormones.

In mammals, the phenotypic development of external and internal reproductive structures in the male is dependent on the hormones the male gonad produces after it has formed (Hughes, 2001; Sharpe & Skakkebæk, 2008). In the rat, it has been shown that there is an early time window, called the masculinization programming window (MPW) in which sufficient androgen action is necessary to set up later masculinization of the reproductive tract and guarantee its proper function later in life (Welsh *et al.*, 2008). Since there is a critical window for hormone action, a risk of disruption if external interferences occur should be considered (Sharpe, 2006). For that reason, reproductive development disorders in males can be caused by deficient action of androgens within the MPW (Sharpe, 2012) and may serve as an explanation for the increasing incidence of reproductive disorders in men. It was proposed that male disorders such as low sperm counts, cryptorchidism, hypospadias and testicular cancer could have a common origin in fetal life, in which testicular dysgenesis results in impairment of fetal testis somatic cell function (Skakkebæk *et al.*, 2001, 2006). This is named the Testicular Dysgenesis Syndrome (TDS) and it is believed that the malformed, or dysgenic, fetal testis will cause faulty Leydig and/or Sertoli cells function, increasing the risk of reproductive disorders (Figure 3; Sharpe & Skakkebæk, 2008). TDS shows how important the normal development of the testis is, along with normal hormone action, for normal reproductive function/health in adulthood (Sharpe, 2006).

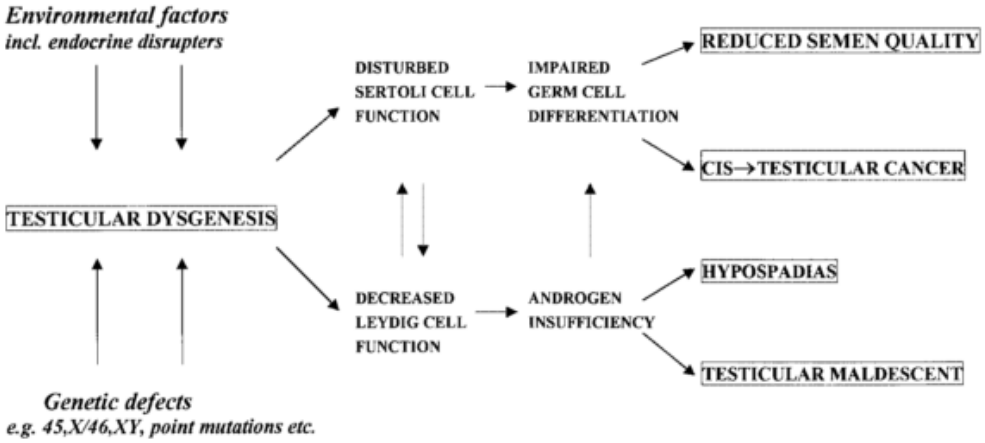


Figure 3. Schematic representation of Testicular Dysgenesis Syndrome, where early disruption of normal testis development due to genetic or environmental factors can result in clinical problems later in life [Image reproduced from Skakkebæk *et al.*, 2001]

Testicular Germ Cell Cancer

The normal development of the testis, as previously shown, can be disturbed by environmental factors, leading to impaired germ cell differentiation and resulting in testicular cancer. Testicular germ cell cancer (TGCC) accounts for 1-2% of all cancers in men, but it is the most common type of cancer in early adulthood, being the biggest cause of cancer-related death in 15-35 year-old males (Toppari *et al.*, 1996; Sharpe & Skakkebæk, 2003). TGCC incidence has doubled in the past 40 years and, fortunately, has a high rate of treatment success (Horwich *et al.*, 2006; Bosl & Motzer, 1997).

Normal germ cell development during the fetal period is essential to guarantee normal functional sperm during adult life. As said previously, highly proliferative and undifferentiated primordial germ cells migrate to the genital ridge and are enclosed by Sertoli cells, which begin an ongoing differentiation, during fetal life. The primordial germ cells are then called gonocytes and begin to differentiate into spermatogonia, the initial cell type of the spermatogenesis process, during the 2^{nd} and 3^{rd} trimester of pregnancy. The remaining gonocytes disappear throughout the first year of postnatal life (Skakkebæk *et al.*, 1998) and normal spermatogenesis can occur in adulthood (Sharpe, 2010). During fetal rat development, primordial germ cells only proliferate during their pluripotency phase, typified by the expression of pluripotency markers shared with embryonic stem cells, such as octamer-binding transcription factor 3/4 (OCT3/4). The loss of this marker coincides with the quiescence phase of germ cells. In the human, germ cells have a similar behaviour, where they also lose OCT3/4 expression (Cools *et al.*, 2006), though differentiated germ cells (OCT3/4 negative germ cells) still proliferate at a much lower rate (Mitchell *et al.*, 2010). When this happens, they switch on expression of VASA and MAGE-A4 which mark the transition to a differentiated, and still slightly proliferative, state (Rajpert-De Meyts, 2006).

The loss of pluripotency of primordial germ cells holds the hypothesis of how TGCC begins in humans. If the primordial germ cells fail to lose pluripotency (Horwich *et al.*, 2006; Western *et al.*, 2010) or there is a delay or block in their development (Cools *et al.*, 2006), they can enter an arrested state where they tend to be more proliferative than normal differentiated germ cells. This is believed to lead to the development of carcinoma-*in-situ* (CIS) cells (Rajpert-de Meyts & Hoei-Hansen, 2007; Sharpe, 2010; Western *et al.*, 2010), which are germ cells with pluripotency and proliferative features that can spread along a seminiferous tubule throughout childhood, probably leading to TGCC in young adulthood (Sharpe, 2010). Support for this theory has been given by Jørgensen *et al.* (1995), who showed that CIS cells express a similar profile of markers as do gonocytes and primordial germ cells, having stem cell-like features such as pluripotency that are down-regulated when they differentiate into spermatogonia (Rajpert-de Meyts & Hoei-Hansen, 2007) (Figure 4). It has been hypothesized that impaired function of supporting cells (Sertoli, Leydig and

Peritubular Myoid cells) alters normal germ cell differentiation, as conditions of partial androgen insensitivity are associated with substantially increased risk of TGCC (Skakkebæk *et al.* 1998; Eddy, 2002; Sonne *et al.*, 2009; Wohlfahrt-Veje *et al.*, 2009). Because these events happen during pregnancy, it suggests that there can be environmental or lifestyle effects on the foetus via the mother that are likely to be irreversible (Sharpe, 2010).

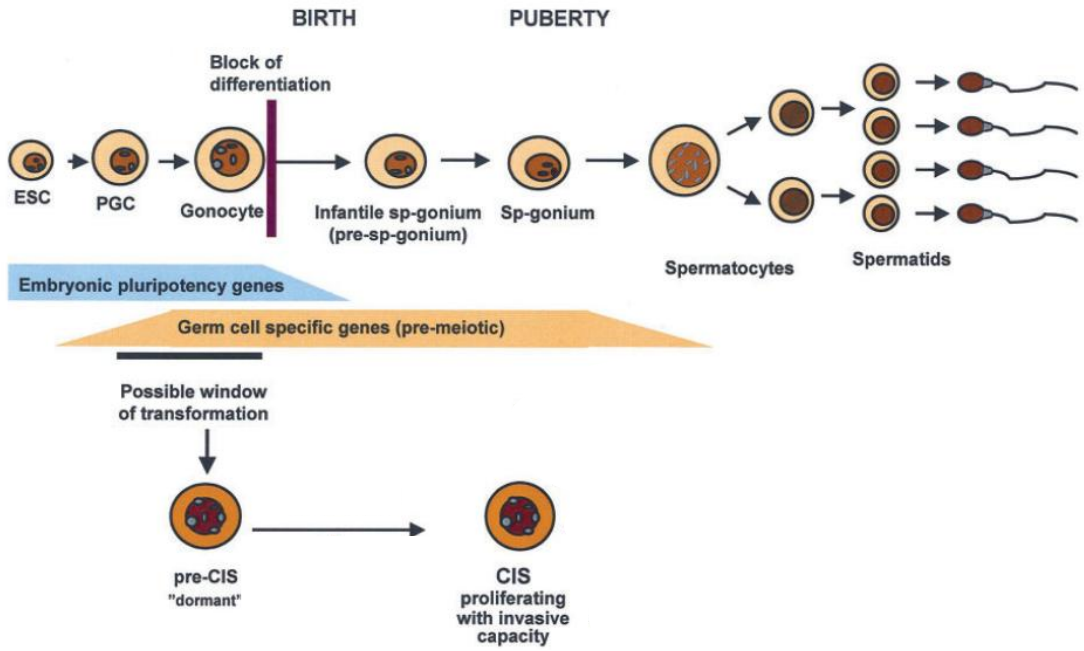


Figure 4. Current hypothesis of how CIS cells are thought to arise, because of a failure of primordial germ cell/gonocyte differentiation, which would normally result in the loss of pluripotency gene expression. The resulting CIS cells stay dormant until puberty and then develop into a tumour in young adulthood. ESC, embryonic stem cells; PGC, primordial germ cells; CIS, carcinoma *in situ* cells. [Image reproduced and adapted from Rajpert-De Meyts, 2006].

When it comes to environmental chemicals that might affect testis development, they are considered a growing threat to health due to their wide use in developed countries. Occupational exposure and pollutants have been related to negative effects on sperm counts and spermatogenesis in the human (Sharpe, 2010). Additionally, studies in animal models showed that environmental chemical exposure, either to individual chemicals (although in considerably higher doses than expected to affect humans) or to a combinations of several chemicals, due to lifestyle choices, can induce disorders similar to those of TDS in offspring (Gray *et al.*, 2001; Sharpe, 2012). This study focuses on how environmental chemicals, namely phthalates and indomethacin, have an impact on germ cell development in the human and rat fetal testis.

Phthalates

Phthalates are the most abundant man-made environmental chemicals, used extensively in industrial applications, such as solvents, plasticizers, floorings, medical devices, personal-care and cosmetic products and others. The routes of intake include ingestion, inhalation and dermal contact (Diamanti-Kandarakis *et al.*, 2009). Phthalate exposure in fetal life has been shown to be associated with reduced anogenital distance, cryptorchidism, hypospadias and decreased fetal testis testosterone synthesis in rats (Fisher, 2004; Diamanti-Kandarakis *et al.*, 2009; Drake *et al.*, 2009). It is important to note that most experimental studies have used the rat as a model and have used phthalate exposure levels far higher than occur in humans and, consequently, extrapolation to the human situation should be done carefully. There are many different phthalates and not all of them cause effects on reproductive development in rats (Gray *et al.*, 2001). The most common phthalates that humans are exposed to are diethylhexyl phthalate (DEHP) and di(*n*-butyl) phathalate (DBP), both of which have been shown to have similar effects on the fetal rat testis (Howdeshell *et al.*, 2008). Both of these phthalates are metabolised to their monoesters, mono-(2-ethylhexyl) phthalate (MEHP) and monobutyl phthalate (MBP) respectively, which act as active toxicants in the testis (Fisher, 2004).

Phthalates have been used in different animal models to show how they can disrupt the normal development of the reproductive tract. Higuchi *et al.* (2003) showed that DBP caused reproductive toxicity in rabbits, with a more pronounced effect if the exposure was in *utero*. Impairment of spermatogenesis and long-term maldevelopment of the male reproductive structures after in *utero* exposure to DBP in rats was documented by Barlow & Foster (2003). Lee & Veeramachaneni (2005) used low concentrations of DBP during the sexual differentiation period on *Xenopus laevis* frogs, which revealed a variety of testicular lesions that persisted into adulthood, long after the exposure to DBP had ceased. It was proposed that dermal contact, due to living in an aquatic environment, could have enhanced the DBP effects, which could mimic the exposure of mammals in amniotic fluid. Other studies in rodents concluded that fetal exposure to DBP impaired normal seminiferous cord development, with an increased incidence of multinucleated gonocytes (Ferrara *et al.*, 2006; Gaido *et al.*, 2007; Scott *et al.*, 2007). *In utero* exposure to DBP also has effects on germ cell differentiation in rats, causing a slight prolongation of pluripotency marker expression and other effects indicative of “delayed” normal development (Ferrara *et al.*, 2006; Jobling *et al.*, 2011). This can be compared with the idea that CIS cells arise from arrested gonocytes, maintaining pluripotency gene expression and behaviour that enhances their potential to become malignant.

Although phthalates act in an anti-androgenic way by suppressing fetal Leydig cell androgen production, their effects on germ cells are independent of androgen action

(Lehraiki *et al.*, 2009) and therefore probably involve alteration of Sertoli cell function (Li *et al.*, 2000; Jobling *et al.*, 2011). Therefore, because embryonic development has conserved pathways, altered germ cell development and induction of TDS-like disorders after phthalate exposure in rats points to potential effects also in humans (Fisher, 2004; Carruthers & Foster, 2005; Kleymenova *et al.*, 2005; Gaido *et al.*, 2007). Indeed, Lambrot *et al.* (2009) have reported an effect *in vitro* of phthalates on germ cell number in the fetal human testis. In addition, Fisher (2003) concluded, after analysing the effects of DBP exposure *in utero* on rat testis development, that the results were remarkably similar to human TDS disorders and, since there is no animal model for human TDS currently, studying fetal rat development could provide a model system to understand the underlying mechanisms of human TDS. However, Mitchell *et al.* (2012) reported that human fetal testes, which were xenografted subcutaneously under the dorsal skin of male nude mice, appear to have unaffected steroidogenesis after the host mice are treated with DBP. This is in accordance with previous *in vitro* data and marmoset studies (Lambrot *et al.*, 2009; McKinnell *et al.*, 2009).

However, we still do not know if the level of phthalate exposure in humans is sufficient to impair testis development and if *in utero* exposure can affect germ cells during fetal development.

Paracetamol effect through Prostaglandins

Paracetamol is generally used during pregnancy, which is believed to be safe at therapeutic doses, along with other over-the-counter painkillers (Werler *et al.*, 2005), in response to inflammatory processes such as pain. However, the use of analgesics during pregnancy has been associated with increased risk of asthma (Rebordosa *et al.*, 2008) and cryptorchidism (Kristensen *et al.*, 2011a) in children. Paracetamol is believed to work through the inhibition of prostaglandins, although the exact mechanism of action is still unknown. Prostaglandins are short-lived lipids that are synthesized by many cells (excluding mature erythrocytes) and are released as a response to cell membrane traumas such as inflammation. Nonsteroidal anti-inflammatory drugs (e.g., paracetamol, indomethacin and aspirin) seem to act through the inhibition of prostaglandin release in response to inflammatory signals, thus relieving pain (McDonald-Gibson & Collier, 1979; Vane & Botting, 1987). Prostaglandins have arachidonic acid as a precursor, the production of which is catalyzed by cyclooxygenase (COX), which has two isoforms: COX-1 and COX-2. COX has three different folding units, an epidermal growth factor-like domain, a membrane-binding domain and an enzymatic domain. The COX active site presents itself as a channel, with a hydrophobic profile. COX-2 can bind larger molecules than COX-1, due to its

bigger active site (Vane & Botting, 1998). Paracetamol is believed to compete with arachidonic acid for the COX binding site, with some preference for COX-2, hence inhibiting the formation of prostaglandins and reducing the inflammatory response (FitzGerald, 2003; Graham & Scott, 2003) (Figure 5). Indomethacin works by inhibiting directly and non-selectively the catalytic action of COX (although it is more active against COX-1), binding to the upper portion of the active site, preventing its substrate, arachidonic acid, from entering the active site and preventing the formation of prostaglandins (Mitchell *et al.*, 1994). It is considered to be a more unequivocal suppressor of prostaglandins that paracetamol.

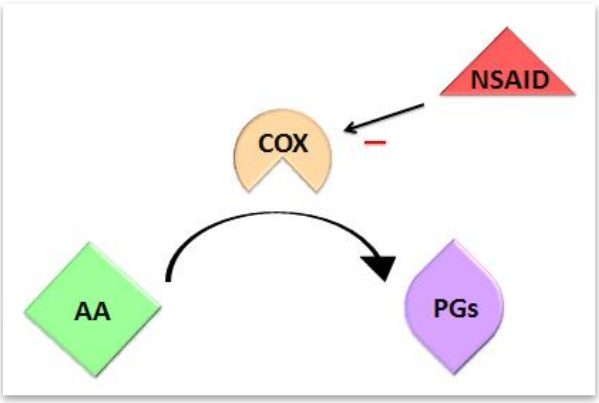


Figure 5. Formation of prostaglandins (PGs) after arachidonic acid (AA) is catalysed by cyclooxygenase (COX). It is believed that nonsteroidal anti-inflammatory drugs (NSAID), like paracetamol and indomethacin, inhibit PGs production by competing with AA for the COX binding site.

Paracetamol exposure in pregnant rats has been shown to cause mild inhibition of testosterone and prostaglandin D2 production (Kristensen *et al.*, 2011a). Older studies have suggested a link between prenatal exposure to mild analgesics and reduced masculinisation in animals (Gupta & Goldman 1986; Gupta, 1989). This is further supported by the anti-androgenic effects of paracetamol discovered in rat models leading to a small reduction in anogenital distance (Kristensen *et al.*, 2011a). As prostaglandin receptors are also expressed in Sertoli cells, contributing to cell differentiation and expression of male specific genes in mice (Wilhelm *et al.*, 2005), it is possible that disruption of this system could be caused by exposure to paracetamol or other painkillers during pregnancy, such as indomethacin.

Aims

This study is focused on germ cell development during fetal life and on the impact of chemical/medicinal drug exposure, both in the human and in the rat. Therefore, it is my aim to: (1) identify whether DBP exposure affects male human fetal germ cell development and differentiation in a xenograft model; and (2) to analyze the effect of paracetamol and indomethacin on male rat germ cell number. Both studies have been prompted due to preliminary unpublished data developed by Lenka Hrabalkova (2011).

Protocols and Procedures

Human studies

Tissue collection and xenografting procedure

Second-trimester (14 – 20 weeks, *n* = 8) human fetal testes were obtained following termination of pregnancy, which were unrelated to fetal abnormalities. Women gave consent in accordance with British national guidelines and ethical approval was obtained from the Lothian Research Ethics Committee, as reported by Mitchell *et al.* (2010). Small portions of each testis were fixed as a pre-graft control. The rest were handled for xenografting, by being placed in ice cold media (containing Liebowitz L-15 with glutamine, 10% fetal bovine serum, 1% penicillin/streptomycin and 1% non-essential amino acids; all Sigma, Poole, UK). Fetal testis specimens were also obtained at gestational ages equivalent to the end of the grafting period and fixed as age-matched controls. Xenografting followed the protocol described in Mitchell *et al.* (2010; Figure 6), which was shown to recapitulate normal fetal testis growth for 6 weeks or more after grafting. After xenografting 4 – 6 human fetal testis pieces under the back skin of castrate adult male nude mice, and allowing 7 days for grafts to establish a blood supply, the host mice were treated daily by oral gavage with 500mg/kg/day of DBP, MBP or vehicle (control) for a period of 21 days. Additionally, they received injections of 20 IU of hCG three times a week in order to replicate normal pregnancy conditions and to maintain testosterone production, as described in Mitchell *et al.* (2010). Overall, 32 samples were analyzed (from each of eight foetuses, there were at least 2 vehicle and 2 DBP/MBP-exposed xenografts).

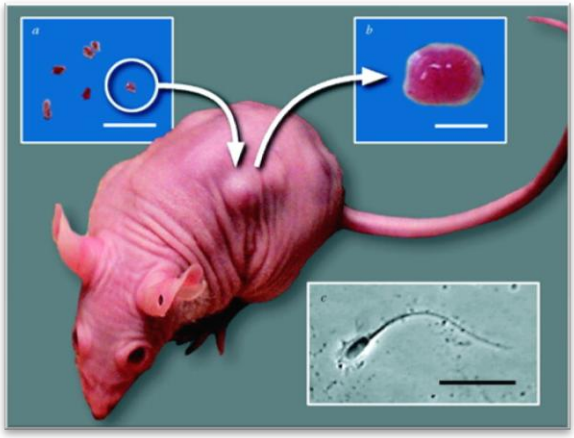


Figure 6. Image representative of the xenografting procedure. Small pieces of donor human testis tissues are inserted subcutaneously under the dorsal skin of castrated male CD1 nude mice. After the post-surgery, mice are exposed to the treatment explained in the text. Grafts were retrieved and weighted after 6 weeks.

Tissue fixation, microtoming and mounting

Pre-grafted, xenografted and age-matched controls and xenografted treated tissues were fixed in Bouin's fixative for two hours, transferred to 70% ethanol and then processed into paraffin blocks using standard procedures.

For microtoming, all paraffin blocks were chilled by being placed on a metal plate on top of a container full of ice. Blocks were trimmed and cut into 5µm sections on a manual rotative microtome (Leica RM 2135; Leica Microsystems), forming wax ribbons. These were transferred to a 40°C waterbath (Tissue Flotation Bath; ThermoScientific) and then placed on electrostatically positive charged slides (Surgipath X-Tra Adhesive Precleaned Microslides; Leica Microsystems), which were left overnight at 57°C in a fanned convection incubator (Carbolite, UK).

Triple Immunofluorescence for OCT3/4, MAGE-A4 and Ki67

Markers for undifferentiated germ cells (octamer binding transcription factor 3/4; OCT3/4), differentiated germ cells (MAGE-A4) and proliferating cells (Ki67) were used to investigate germ cell expression patterns, as per previous reports (Gaskell *et al.*, 2004; Mitchell *et al.*, 2008; Mitchell *et al.*, 2010). Information regarding antibodies, dilutions, sera and fluorescence used are shown in Table I.

Sections were subjected to a dewax and rehydration process, by immersing in xylene (5 min), then in graded alcohols (100%, 90% and 70%, 20sec each) and finally washed in tap water. Heat-induced antigen retrieval was performed in 0,01M citrate buffer (citrate is diluted in deionized water, 1:10; pH6; Sigma) and, after being left to cool in tap water, the slides were subjected to an endogenous peroxidase block with 3% (v/v) H_2O_2 (VWR Prolabo) in methanol (Fisher Scientific, UK) for 30min. Two 5min TBS (Tris-buffered saline; 0,05M Tris and 0,85% NaCl; pH 7,6) washes were performed, and they were repeated between each step of this immunofluorescence protocol. Slides were then incubated for 30min in normal chicken serum diluted 1:5 with TBS containing 5% (w/v) bovine serum albumin (NChS/TBS/BSA). Slides were then incubated overnight (at 4°C) with primary antibody OCT3/4.

Table I. Antibodies, dilutions, sera and fluorescence details for the triple immunofluorescence procedure on human fetal testis. [a] Gift Dr Guilio Spagnoli; [b] Chicken anti-Goat Peroxidase; [c] Chicken anti-Mouse Peroxidase; [d] Normal Chicken Serum; [e] Tyramide-Cyanine3; [f] Tyramide-Cyanine5; [g] Tyramide-Fluorescein. All secondary antibodies were diluted 1:200 in NChS/TBS/BSA.

Primary Antibody	Source	Species Raised	Dilution	Secondary Antibody	Normal Serum/TBS/BSA	Fluorescence
OCT3/4	Santa Cruz	Goat	1:100	ChaGP[b]	NChS[d]	Tyramide-Cy3[e]
MAGE-A4	Gift[a]	Mouse	1:100	ChaMP[c]	NChS	Tyramide-Cy5[f]
Ki67	Dako	Mouse	1:200	ChaMP	NChS	Tyramide-Fl[g]

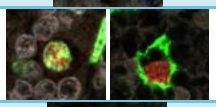
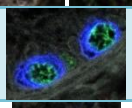
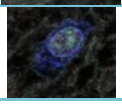
On the next day, slides were incubated with secondary antibody ChaGP for 30min, according to Table I. Then, Tyramide-Cy3 (Tyramide Signal Amplification™ – Cyanine3; PerkinElmer), which is red detection, was applied for 10min to all slides following the manufacturer's instructions. Slides, which were kept in the dark from this point onwards, were blocked with 3% (v/v) H_2O_2 in TBST (TBS plus 0.05% Tween; Tween®20, Sigma). Normal chicken serum block step was repeated and MAGE-A4 primary antibody was applied to all slides, which were left to incubate overnight (at 4°C).

The next day, slides were submitted to secondary antibody ChaMP for 30 min (see Table I). They were then submitted to Tyramide-Cy5 (blue detection; Tyramide Signal Amplification™ – Cyanine5; PerkinElmer) for 10 min. After this, slides were microwaved in citrate buffer (with same dilution as for the heat-induced antigen retrieval step) on full power for 2.5min and then left to stand for 20min. Normal chicken serum block was repeated once again and slides were then incubated overnight (at 4°C) with the last primary antibody (Ki67). Afterward, secondary antibody ChaMP was applied the next day for 30 min. Slides were then incubated with Tyramide-Fl (green detection; Tyramide Signal Amplification™ – Cyanine5; PerkinElmer) for 10min. To stain all cell nuclei, all slides were counterstained for 10 min with DAPI (4',6-diamidino-2-phenylindole; Sigma), which is diluted 1:1000 in TBS. Permafluor Aqueous Mounting Medium (ThermoScientific) was used to mount the slides.

Fluorescence capture and germ cell quantification

Fluorescence was captured using a Zeiss LSM 710 Axio Observer Z1 confocal laser microscope (Carl Zeiss Ltd.), which scans individual tiles from the full section, without overlap. These tiles were saved and used to count stained germ cells according to Table II. Since Ki67 is not specific for germ cells, it was not considered unless it co-localized with either OCT3/4 and/or MAGE-A4 staining. Overall, there were six different staining classes (Table II):

Table II. List of all the staining combinations counted after scanning fetal human testis immunofluorescently for three different antibodies (OCT3/4, MAGE-A4, Ki67). Ki67 is a proliferation marker and it is not germ cell specific, so it was only recorded when in combination with either of the other two germ cell specific markers.

Germ cell antibodies	Detected germ cell fluorescence	Real Example	Stained cell compartment
OCT3/4	Red		Nucleus
MAGE-A4	Blue		Cytoplasm
MAGE-A4 + OCT3/4	Red + Blue		Nucleus and cytoplasm
Ki67 + OCT3/4	Green + Red		Only nucleus or cytoplasm and nucleus
Ki67 + MAGE-A4	Green + Blue		Nucleus and cytoplasm
Ki67 + MAGE-A4 + OCT3/4	Green + Blue + Red		Nucleous and cytoplasm

All of these combinations were counted manually with a "laboratory counter" (Clay Adams Laboratory Counter). Then, the percentage of OCT3/4 positive (OCT3/4^{+}) and MAGE-A4 positive (MAGE-A4^{+}) cells were calculated, in order to know the fraction of proliferating cells (cells stained for OCT3/4, or MAGE-A4, together with Ki67). Once the information for each age was gathered, average means were calculated to be statistically analysed. For the grafts numbered FT2546/7 (17w/19w), two foetuses were grafted but they were indistinguishable, so they were handled as an 18w foetus. MBP was used in some cases and is considered as being equivalent to DBP.

Rat Studies

Tissue collection, fixation, microtoming and mounting

Wistar rats were housed under standard conditions according to UK home office guidelines and had free access to fresh tap water and soy-free food. Time-mated female rats were treated daily with paracetamol (350mg/kg/*day*) by oral gavage or with indomethacin (1mg/kg/dia or 0.8mg/kg/dia) dissolved in corn oil by subcutaneous injection. Control females were treated with corn oil by oral gavage or subcutaneous injection. Table III shows the gestational ages when the treatments were performed:

Table III. Treatment details during rat fetal development regarding the testis collection day (e, embryonic day; pnd, postnatal day).

		Treatment Period		
		Control	Paracetamol	Indomethacin
Testis Collection Day	e21.5	e15.5 – e18.5 (*n* = 12, from 8 litters)	e15.5 – e20.5 (*n* = 9, from 5 litters)	e15.5 – e18.5 (*n* = 14, from at least 6 litters)
	pnd25	e15.5 – e18.5 (*n* = 4, from 4 litters)	–	e15.5 – e18.5 (*n* = 4, from at least 3 litters)

Tissue collection and fixation was performed in accordance with Dean *et al.*, 2012. To generate slides, the microtoming and mounting technique used previously for human tissue was replicated for rat tissue, with no modifications.

Immunohistochemistry for VASA

Antibody dilutions and serum used are listed in Table IV. Tissue dewax and rehydration, antigen retrieval, methanol/peroxide block and TBS washes were all done as described above for triple immunofluorescence in human testis, with no modifications. For normal serum block, normal goat serum (NGS/TBS/BSA; dilution performed as explained for NChS/TBS/BSA) was used to incubate slides for 30min. Then, primary antibody VASA, a germ cell specific marker (Rajpert-De Meyts, 2006), was added to slides and left overnight (at 4°C).

Table IV. Antibody, dilution and serum details for the immunohistochemistry procedure on rat testis. [a]Goat anti-Rabbit Biotinylated (diluted 1:500 in NGS); [b] Normal Goat Serum.

Primary Antibody	Source	Species Raised	Dilution	Secondary Antibody	Normal Serum/TBS/BSA
VASA	Abcam	Rabbit	1:20	GaRB[a]	NGS[b]

The next day, after two 5min TBS washes, slides were incubated for 30 min with secondary antibody, diluted 1:500 in NGS. Rat testis sections were then incubated with Streptavidin labelled horseradish peroxidase enzyme conjugate (Streptavidin-HRP; Vector laboratories), diluted 1:1000 in TBS, for 30min. Staining was then developed using 3,3-diaminobenzidine tetrahydrochloride (DAB substrate kit for peroxidise; Vector laboratories) for up to 10min. This reaction was stopped by placing the slides in tap water and there was no need to perform any other TBS wash. The slides were counterstained with haematoxylin and washed in tap water. They were drained as much as possible and submitted to a dehydration process, by being exposed to graded alcohols (70%, 80%, 90%, 100%; 20sec each). Then, the slides were placed in xylene (5min) and mounted using a xylene-based mountant, Pertex (Cellpath plc).

Staining detection and germ cell quantification

All images were captured using a JVC camera attached to an Olympus AX70 microscope and all countings were performed using Axiovision Rel. 4.8. software.

Germ cell quantification followed a stereology method, which is a technique that extracts quantitative information, such as the cellular composition, of a three-dimensional tissue from measurements made on two-dimensional planar sections of that tissue. The principle relies on identifying how many cell nuclei are present, as most nuclei are essentially spheroidal and thus the chances of detecting a cell nucleus is directly proportional to the number of such nuclei and their size. Stereology aims to estimate the number of cell nuclei and then, after measuring average nuclear size, to determine the absolute numbers of that cell in a given 2-dimensional area of tissue. The number of such cell per whole organ/tissue specimen can then be determined if

the weight or volume of the specimen is known. Stereology does not just count all cell nuclei, it does this in an “unbiased” manner, either by random selection of areas to be counted or by systematic selection. On fields of view, a grid with 25 x 19 points is then superimposed and the percentage of points that fall over the nucleus in question is counted. This enabled the determination of the total germ cell nuclear volume per testis. After this, germ cell mean nuclear volume was measured following the same stereology principles (by measuring the volume of germ cell nucleus from a random selection of two-dimensional fields, which is assumed to be representative of the three-dimensional tissue, and then by calculating the mean volume of all measured germ cell nuclei) (Figure 7).

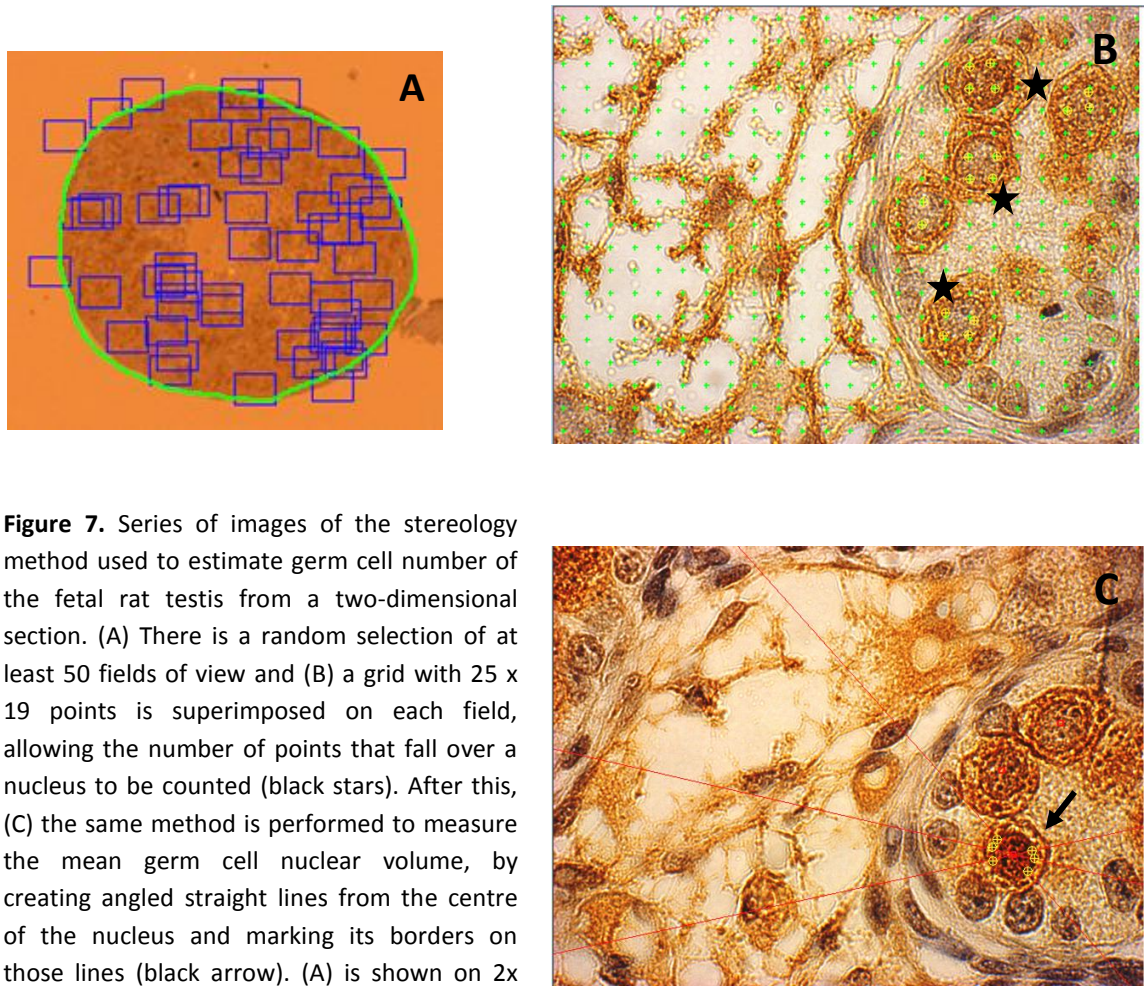


Figure 7. Series of images of the stereology method used to estimate germ cell number of the fetal rat testis from a two-dimensional section. (A) There is a random selection of at least 50 fields of view and (B) a grid with 25 x 19 points is superimposed on each field, allowing the number of points that fall over a nucleus to be counted (black stars). After this, (C) the same method is performed to measure the mean germ cell nuclear volume, by creating angled straight lines from the centre of the nucleus and marking its borders on those lines (black arrow). (A) is shown on 2x objective; (B, C) are shown on 63x objective.

The percentage of points that fall over a germ cell nucleus and the germ cell mean nuclear volume are used to calculate the number of germ cells (in millions), through the formulas shown in Figure 8 below.

$$\%\ Points = \frac{total\ number\ of\ points\ that\ fall\ over\ a\ GC\ nucleus}{25\ \times 19\ \times nº\ random\ fields\ counted} \times 100$$

$$GC\ Absolute\ Nuclear\ Volume = \frac{\%\ Points\ \times Testis\ Weight\ (mg)}{100}$$

$$GC\ numbers\ (millions) = \frac{GC\ Absolute\ Volume}{GC\ Mean\ Nuclear\ Volume} \times 1000$$

Figure 8. Formulas used to calculate the germ cell number (millions), which was then used in statistical analysis. GC, germ cell.

For each e21.5 section, between 50 – 100 random fields were selected at 2x objective and all measurements were then performed using a 63x objective. All cells stained with VASA within a clearly defined tubule were assumed to be germ cells and counted as such.

For pnd25 comparisons, each section had between 25 – 65 random fields selected at 2x objective and all measurements were then performed using a 63x objective. Germ cells stained with VASA within a clearly defined tubule were separated into three classes, according to the description in Table V. These criteria follow the temporal appearance of different germ cell types with the progression of spermatogenesis.

Table V. All classes of VASA-stained germ cells counted for pnd25 samples, with morphological description.

Germ Cell Class	Description
Spermatogonia	With flat oval nucleus; near or touching the basal membrane
Early Spermatocyte	Round nucleus; close to, but not necessarily touching, the basal membrane
Pachytene Spermatocyte	Big cells with possibly bigger nuclei, darker VASA staining and always inside the early spermatocyte layer, towards the centre of the tubule

Statistical analysis

All statistical analyses were carried out using GraphPad Prism version 5 software and statistical significance was set at $P<0.05$. To analyse the human samples, a paired *t*-test was used after calculating the average means for vehicle-exposed (control) and DBP-exposed samples from the same fetus. This was performed as shown below (Figure 9).

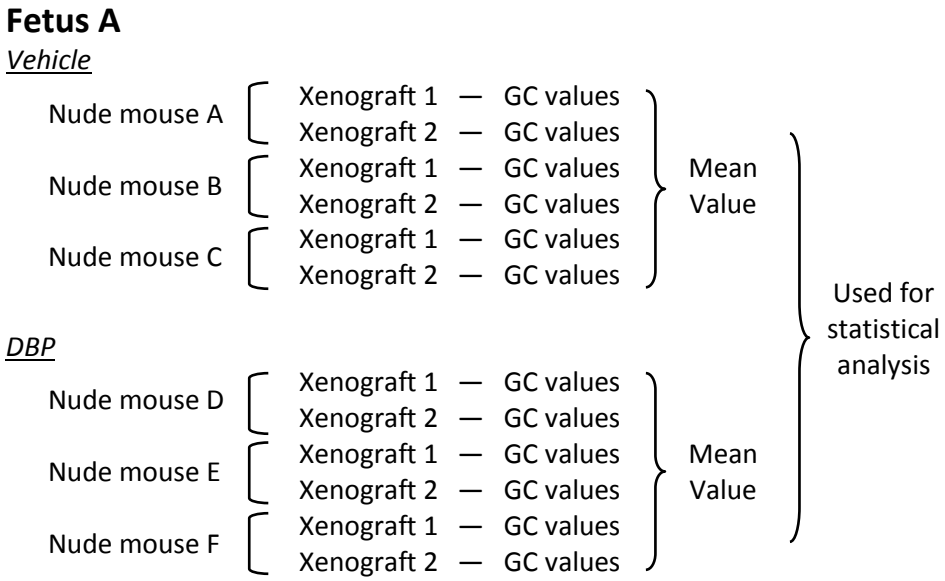


Figure 9. Representation of the statistical analysis of all eight human fetuses. The xenografted pieces were collected from their nude mice hosts and, after counting all types of stained germ cells from that fetus, a mean value was found for: OCT3/4$^+$ germ cells; MAGE-A4$^+$ germ cells; OCT3/4$^+$/Ki67$^+$ germ cells; and MAGE-A4$^+$/Ki67$^+$ germ cells. The control mean value and the DBP-treated mean value for each foetus were then used for statistical analysis. DBP, Di(*n*-butyl) phathalate; GC, germ cell.

For e21.5 rat testis, comparison between control samples with paracetamol and indomethacin-treated samples was done by one-way analysis of variance (ANOVA). To compare pnd25 controls with indomethacin-treated samples, a Student's unpaired *t*-test was performed for each type of germ cell and for the total number of germ cells.

Results

The effect of DBP on germ cell differentiation and proliferation in human fetal testis xenografts

The first step of data analysis was to record the values counted for all six types of stained germ cells, for all of the human fetal testis xenografts for each fetus (Figure 10, red box).

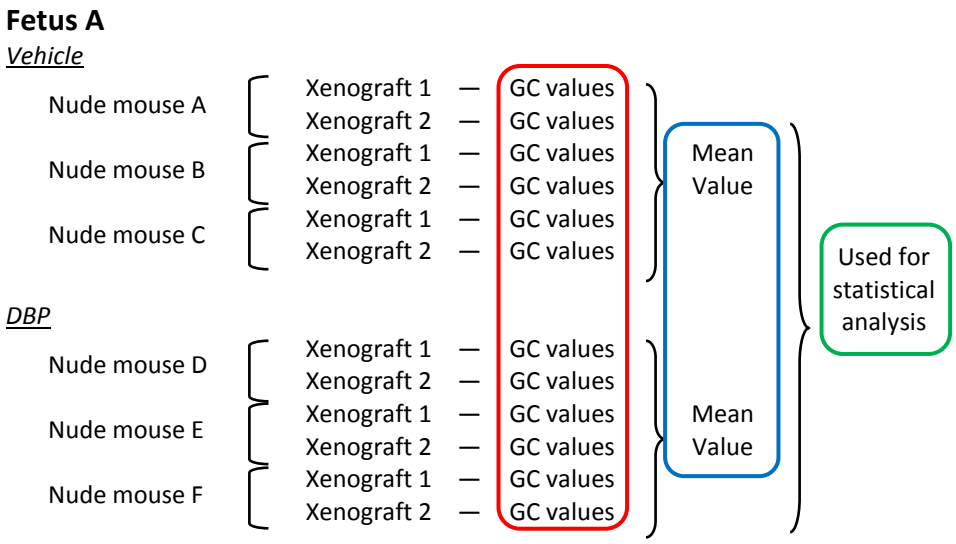


Figure 10. Schematic representation of the first, second and third step of data analysis for the human studies. The first step (red box) consisted of recording the numbers of all six stained germ cell categories counted for all fetuses. The second step (blue box) simply involved calculation of the mean values/fetus for vehicle (control) and DBP-exposed samples for the six GC staining categories. The mean values were then used for statistical analysis (green box). GC, germ cell.

An example of germ cell values recorded for an age-matched control section from a 16 week fetus is shown in Table VI. The same is performed for age-matched controls, pre-grafted controls, vehicle controls and DBP(MBP) exposed xenografts for all foetuses (Table VII). A minimum of 100 germ cells were counted for each human fetal testis xenograft. If that number was not reached with one section, an immunofluorescence protocol was performed on up to 3 sections from the same tissue, until that number was reached, by adding the new values to those previously counted.

Table VI. Germ cell information gathered from a section of an age-matched control of a 16 week foetus (block 2004-1391, section number 17). Germ cell stain types were counted and absolute values were recorded. The total number of germ cells stained for OCT3/4 (and MAGE-A4), whether they were co-stained with Ki67 or not, was calculated. However, because most sections had very low values for $OCT3/4^+$ / $MAGE\text{-}A4^+$ co-stain and triple stain, these values were disregarded when the total $OCT3/4^+$ and $MAGE\text{-}A4^+$ values were determined. Then, the percentage $OCT3/4^+$ germ cells and $MAGE\text{-}A4^+$ germ cells in the section were calculated. The percentage of proliferative cells found in the $OCT3/4^+$ and $MAGE\text{-}A4^+$ pool was also recorded. The highlighted values (red discontinuous line) were the ones used for the next phase of data analysis. MCTRL, age-matched control; %, percentage; GC, germ cells.

Section 2004-1391.17 MCTRL

Germ cell stain types	Absolute values	Percentage (%)	How (%) is called	How (%) was calculated
$OCT3/4^+$	214			
$OCT3/4^+$/ $Ki67^+$	90	29,61	% proliferative $OCT3/4^+$ GC	$\frac{OCT3/4 + Ki67}{Total\ OCT3/4} \times 100$
Total $OCT3/4^+$	304	72,04	% $OCT3/4^+$ GC	$\frac{Total\ OCT3/4}{Total\ GC} \times 100$
$MAGE\text{-}A4^+$	112			
$MAGE\text{-}A4^+$ / $Ki67^+$	5	4,27	% proliferative $MAGE\text{-}A4^+$ GC	$\frac{MAGE\text{-}A4 + Ki67}{Total\ MAGE\text{-}A4} \times 100$
Total $MAGE\text{-}A4^+$	117	27,73	% $MAGE\text{-}A4^+$ GC	$\frac{Total\ MAGE\text{-}A4}{Total\ GC} \times 100$
$OCT3/4^+$ / $MAGE\text{-}A4^+$	0			
Triple	1			
Total GC	422			

Table VII. Germ cell values from all treatments and controls for a 16 weeks foetus. The values highlighted in Table V are shown on the first line of this Table, also highlighted (red discontinuous line). The rest of the table is completed by following the same procedure for all human fetal testis xenograft sections for this 16 week fetus. MCTRL, age-matched control; CTRL, pre-graft control; VEH, vehicle control; DBP (MBP), DBP or MBP exposed samples; GC, germ cell.

16 week fetus	Block	% OCT3/4+ GC	% proliferative OCT3/4+ GC	% MAGE-A4+ GC	% proliferative MAGE-A4+ GC
MCTRL	2004-1391	72,04	29,61	27,73	4,27
CTRL	2010-3588	7,99	8,00	92,01	0,00
VEH	2010-3594 A	8,82	55,56	91,18	0,00
	2010-3594 C	4,55	83,33	95,45	0,00
	2010-3595 A	9,92	58,33	90,08	0,00
	2010-3595 C	2,03	100,00	97,97	0,00
DBP (MBP)	2010-3598 A	4,13	80,00	95,87	0,00
	2010-3598 C	3,13	85,71	96,88	0,46
	2010-3599 A	1,35	100,00	97,97	0,00
	2010-3599 C	5,45	35,71	94,16	2,48

The second step was to find the mean values for control and DBP(MBP) exposed human fetal testis xenografts (Figure 10, blue box). For each fetus, a mean percentage was calculated for control and DBP (MBP) exposed human fetal testis xenografts (Table VIII). All means were gathered into Table IX.

Table VIII. How average means were calculated for control and DBP treated groups following on from the example given for a 16 week fetus in Table VII. The mean percentage of control OCT3/4$^+$ germ cells was found by calculating the average mean of age-matched controls (MCTRL), pre-graft controls (CTRL) and vehicle controls (VEH) (red box). The mean percentage of DBP(MBP) treated OCT3/4$^+$ germ cells was found by calculating the average mean of DBP and MBP exposed xenografts (green box). The same technique was done for % proliferative OCT3/4$^+$ germ cells; % MAGE-A4+ germ cells; % proliferative MAGE-A4+ germ cells. GC, germ cell.

16 week fetus	Block	% OCT3/4$^+$ GC	% proliferative OCT3/4$^+$ GC	% MAGE-A4$^+$ GC	% proliferative MAGE-A4$^+$ GC
MCTRL	2004-1391	72,04	29,61	27,73	4,27
CTRL	2010-3588	7,99	8,00	92,01	0,00
VEH	2010-3594 A	8,82	55,56	91,18	0,00
	2010-3594 C	4,55	83,33	95,45	0,00
	2010-3595 A	9,92	58,33	90,08	0,00
	2010-3595 C	2,03	100,00	97,97	0,00
DBP (MBP)	2010-3598 A	4,13	80,00	95,87	0,00
	2010-3598 C	3,13	85,71	96,88	0,46
	2010-3599 A	1,35	100,00	97,97	0,00
	2010-3599 C	5,45	35,71	94,16	2,48

Table IX. All mean values for all four control and DBP exposed groups (% OCT3/4$^+$ germ cell; % MAGE-A4+ germ cells; % proliferative OCT3/4$^+$ germ cells; % proliferative MAGE-A4+ germ cells). The control mean average (red box) and the DBP (MBP) treated mean average (green box) for the values highlighted in Table VII are shown for a 16 week fetus. These values were used to perform four paired *t*-tests, each one corresponding to one of the analysed groups. w, week; GC, germ cell.

Analysed Groups / Ages	% OCT3/4$^+$ GC		% MAGE-A4$^+$ GC		% proliferative OCT3/4$^+$ GC		% proliferative MAGE-A4$^+$ GC	
	Control	DBP (MBP)	Control	DBP (MBP)	Control	DBP (MBP)	Control	DBP (MBP)
14w	24,63	5,92	74,55	94,08	66,51	55,56	3,21	1,59
16w	17,56	3,51	82,40	96,22	18,80	75,36	0,71	0,74
18w	28,75	2,58	70,96	96,97	57,10	87,50	0,63	0,77
18w	16,75	12,17	82,09	85,67	55,32	86,24	3,55	0,78
19w	5,05	3,69	94,95	96,31	27,24	0,00	3,75	3,79
19w	30,89	29,15	69,11	70,72	87,15	27,17	0,97	0,90
20w	43,78	30,27	56,17	69,58	62,07	67,07	0,78	0,61
20w	18,53	15,06	81,47	84,71	59,38	62,76	1,18	0,14

The statistical analysis (Figure 10, green box) was performed by comparing the values from control xenografts with values from the DBP (MBP) exposed xenografts for each analysed group (% OCT3/4+ germ cells; % MAGE-A4+ germ cells; % proliferative OCT3/4+ germ cells; % proliferative MAGE-A4+ germ cells).

Germ cell differentiation in human fetal testis xenografts was assessed by using a germ cell pluripotency marker (OCT3/4) and a differentiated germ cell marker (MAGE-A4) (Figure 11). Human fetal testis xenografts exposed to DBP (or its active metabolite MBP) showed a decline in the percentage of OCT3/4$^+$ germ cells of more than 10% (paired *t*-test, t=3,25, df=7, p=0,014) (Figure 12, A). In contrast, when comparing the percentage of MAGE-A4$^+$ germ cells, DBP(MBP) treated xenografts show an increase of 10% above control xenografts (paired *t*-test, t=3,14, df= 7, p=0,016) (Figure 12, B).

The proliferation of undifferentiated (OCT3/4$^+$) and differentiated (MAGE-A4$^+$) germ cells was measured using a proliferation cell marker, Ki67, which is non-specific for germ cells (Figure 11). DBP(MBP) xenografts, when compared to control xenografts, showed no difference in the percentage of proliferating OCT3/4$^+$ germ cells, which was ~60% (paired t-test, t=0,27, df=7, p=0,795) (Figure 12, C). The percentage of MAGE-A4$^+$ germ cells also registered no difference between control and DBP(MBP) xenografts, which was ~2% (paired t-test, t=1,84, df=7, p=0,108) (Figure 12 D).

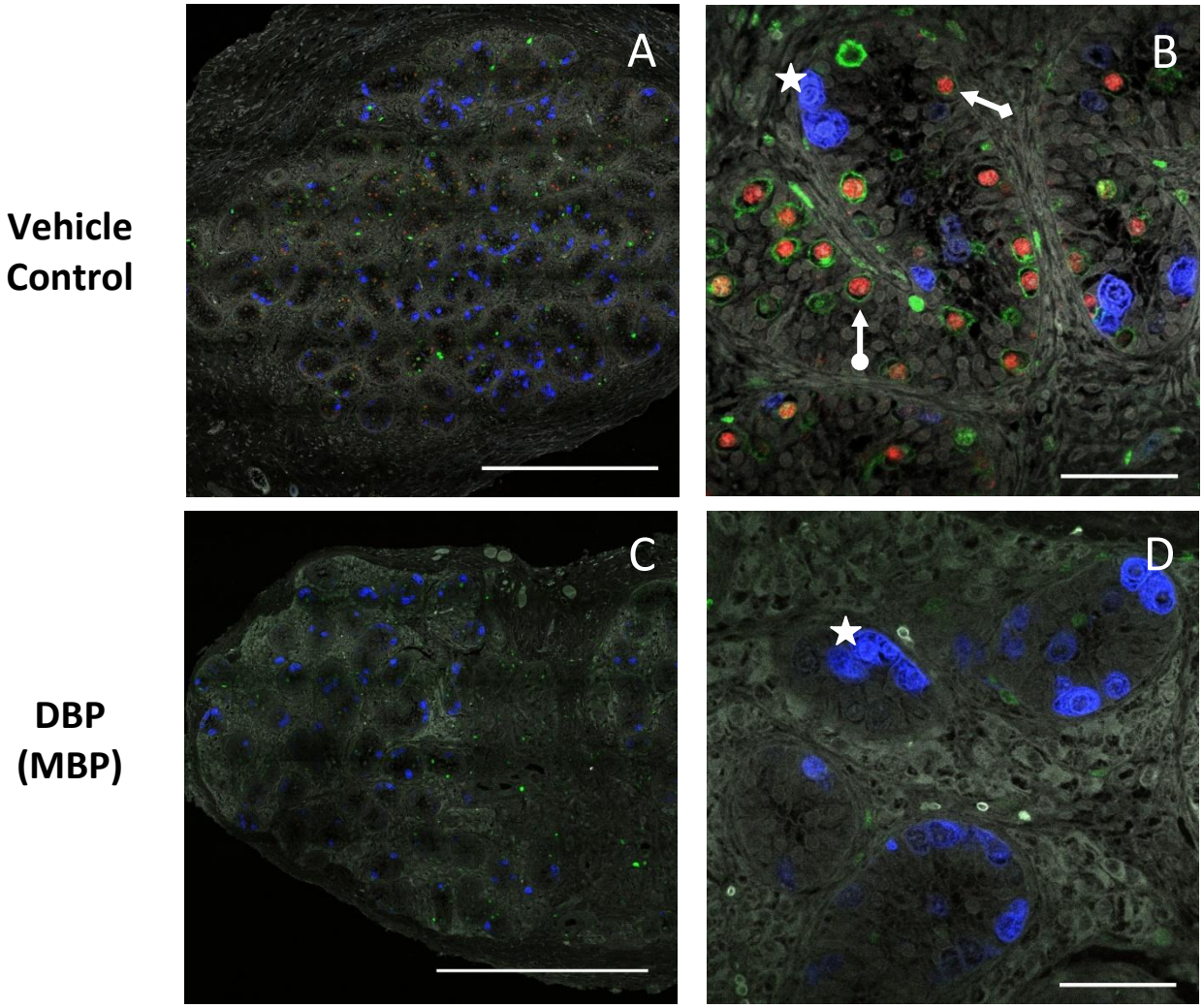


Figure 11. Photographs of vehicle control and DBP(MBP) exposed second trimester human fetal testis xenografts after the immunofluorescence protocol for OCT3/4 (red; square labelled arrow - undifferentiated germ cell marker), MAGE-A4 (blue; star - differentiated germ cell marker) and Ki67 (green; circle labelled arrow - proliferating cell marker, and note that it co-stains with OCT3/4). A, C are confocal images of the testis section that result from overlapped individual tiles. B, D are confocal scanned individual tiles used to count germ cells within a clearly defined seminiferous cord and to assess their proliferation. Partial germ cells were counted only if they were on the left or bottom limit of the tile. Germ cells stained positive for the combinations OCT3/4/MAGE-A4, MAGE-A4/KI67 and OCT3/4/MAGE-A4/Ki67 are not shown. Scale bar (A, C) = 500µm; (B, D) = 50µm.

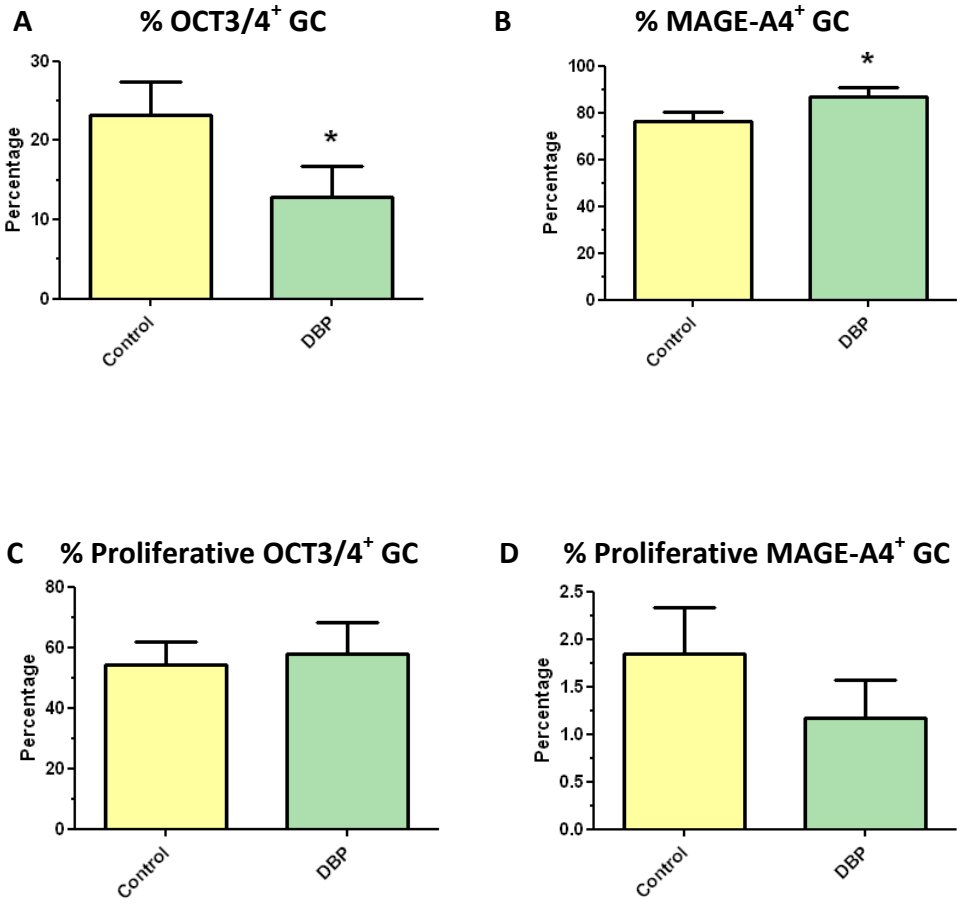


Figure 12. Quantification of germ cell differentiation (A,B) and proliferation (C,D) in control (*n* = 8) and DBP(MBP)-exposed (*n* = 8) second trimester human fetal testis xenografts. 500mg/kg/day of DBP, MBP or vehicle (control) were administered to host male nude mice for a period of 21 days, starting 7 days after xenografting 4-6 human fetal testis pieces under the back skin. Control values include age-matched control, pre-graft control and vehicle control samples. DBP values include DBP or MBP exposed samples. OCT3/4 is a marker for undifferentiated germ cells, MAGE-A4 is a marker for differentiated germ cells and proliferation was assessed using the marker Ki67 (unspecific for germ cell). *p < 0,05, in comparison with respective control value.; GC, germ cell.

The effect of paracetamol and indomethacin on germ cell number in e21.5 rat fetal testes

Germ cell counts were carried out using a stereology method, which calculates the germ cell composition of a three-dimensional tissue (as the fetal testis) by counts performed in two-dimensional sections from that tissue. In e21.5 rat fetal testis, germ cells immunostained with the marker VASA were the only cells considered for counting (Figure 13, A-D). The germ cell absolute nuclear volume and the germ cell mean nuclear volume were used to calculate the total number of germ cells (in millions) present in each testis. After determining the germ cell numbers (in millions) for control, paracetamol and indomethacin exposed fetal testes, comparisons among the three groups were conducted using one-way ANOVA and a Bonferroni's Multiple Comparison. All e21.5 rat testis values (millions) can be found at Appendix.

The number of germ cells in e21.5 testes from indomethacin-exposed fetuses was decreased when compared to controls ($p<0,05$). However, germ cell numbers in e21.5 testes from paracetamol-exposed fetuses were unaffected, comparing with control ($p>0,05$) or the indomethacin exposed group ($p>0,05$), although there was a downward trend (Figure 13, E).

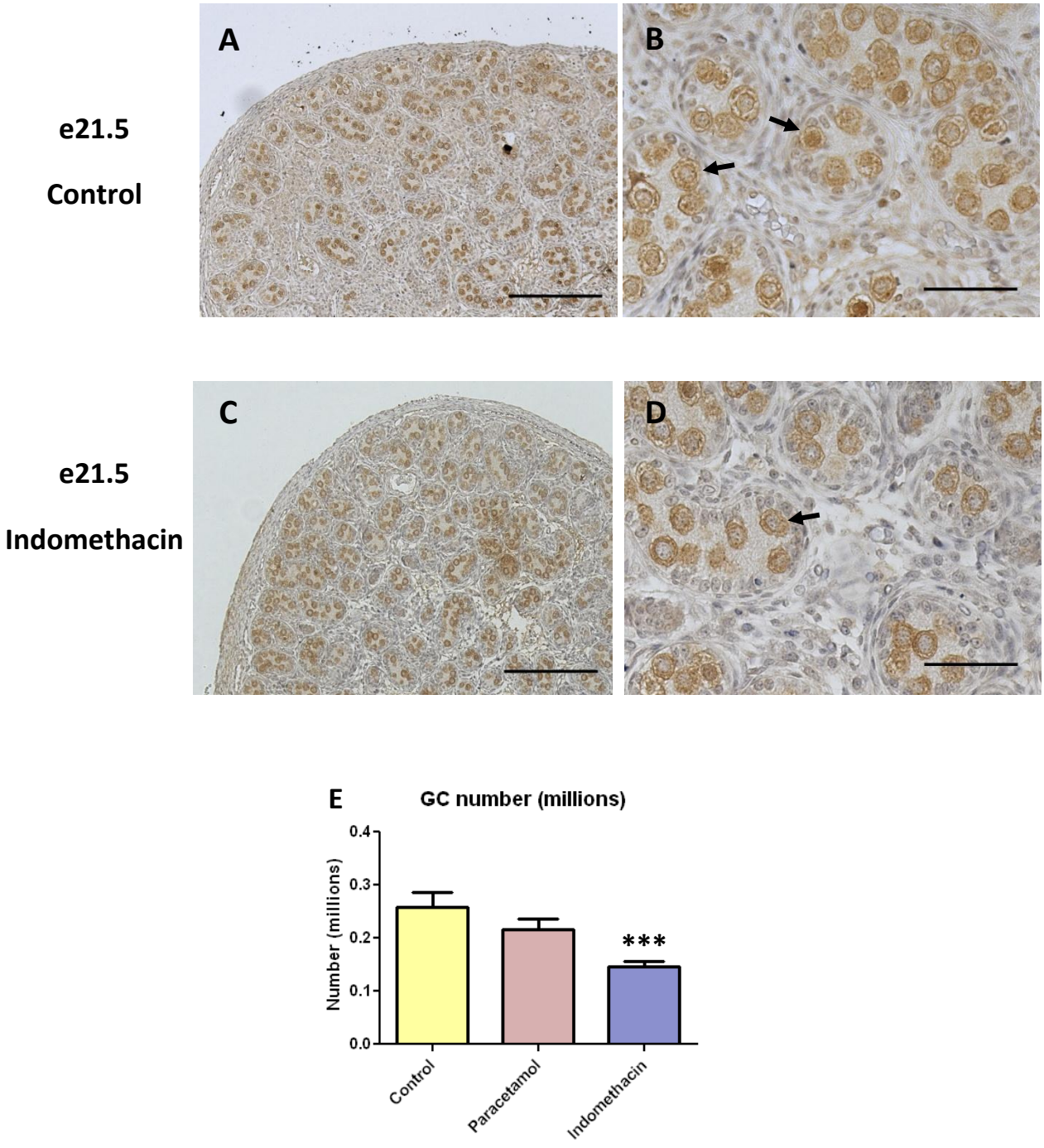


Figure 13. Effect of maternal treatment with paracetamol (350mg/kg/day from e15.5–e20.5) or indomethacin (1mg/kg or 0.8mg/kg from e15.5 – e18.5) on germ cell number in rat fetal e21.5 testes. Germ cells were immunostained for VASA (brown colour, highlighted with black arrows) and stereology used to count the number (millions) of germ cells in control samples (A, B) and in those from indomethacin (or paracetamol)-exposed fetuses (C, D). Scale bar (A, B) = 200µm; (C, D) = 50µm. Values for control (*n* = 12), paracetamol (*n* = 9) and indomethacin (*n* = 14) exposed fetal testes were compared using a one-way ANOVA (E). e, embryonic day; GC, germ cell. *** $p<0.001$, in comparison with control.

The effect of fetal exposure to indomethacin on germ cell number in pnd25 rat testes

To assess if fetal exposure to indomethacin still affected germ cell number after the termination of treatment, germ cells were counted using stereology (as for e21.5 rat fetal testis) in testes from control and indomethacin exposed rats at pnd25. Germ cells were immunostained for VASA and separated into three classes that follow the temporal progression of spermatogenesis: spermatogonia, early spermatocytes and pachytene spermatocytes (Figure 14, A-D). Germ cell absolute and mean nuclear volume was calculated for each class of germ cells and germ cell number (millions) was then calculated. Total germ cell number (millions) for each sample was also recorded by adding all three germ cell class numbers for that sample. All pnd25 rat testis values (millions) can be found in Appendix. A Student's unpaired *t*-test was used to compare the numbers for the different germ cell classes and the total germ cell numbers.

Spermatogonial numbers (millions) were more than double in pnd25 testes from indomethacin-exposed rats, when compared to pnd25 controls (t=3,48; df=6; p=0,013) (Figure 15, A). Early and pachytene spermatocyte numbers (millions) were also increased, but this did not reach statistical significance (Early spermatocytes: t=1,69; df=6; p=0,142; Pachytene spermatocytes: t=0,58; d=6; p=0,583) (Figure 15, B, C). When the germ cell data were added together, the total germ cell number was significantly increased in indomethacin exposed animals, compared to controls (t=2,66; df=6; p=0,037) (Figure 15, D).

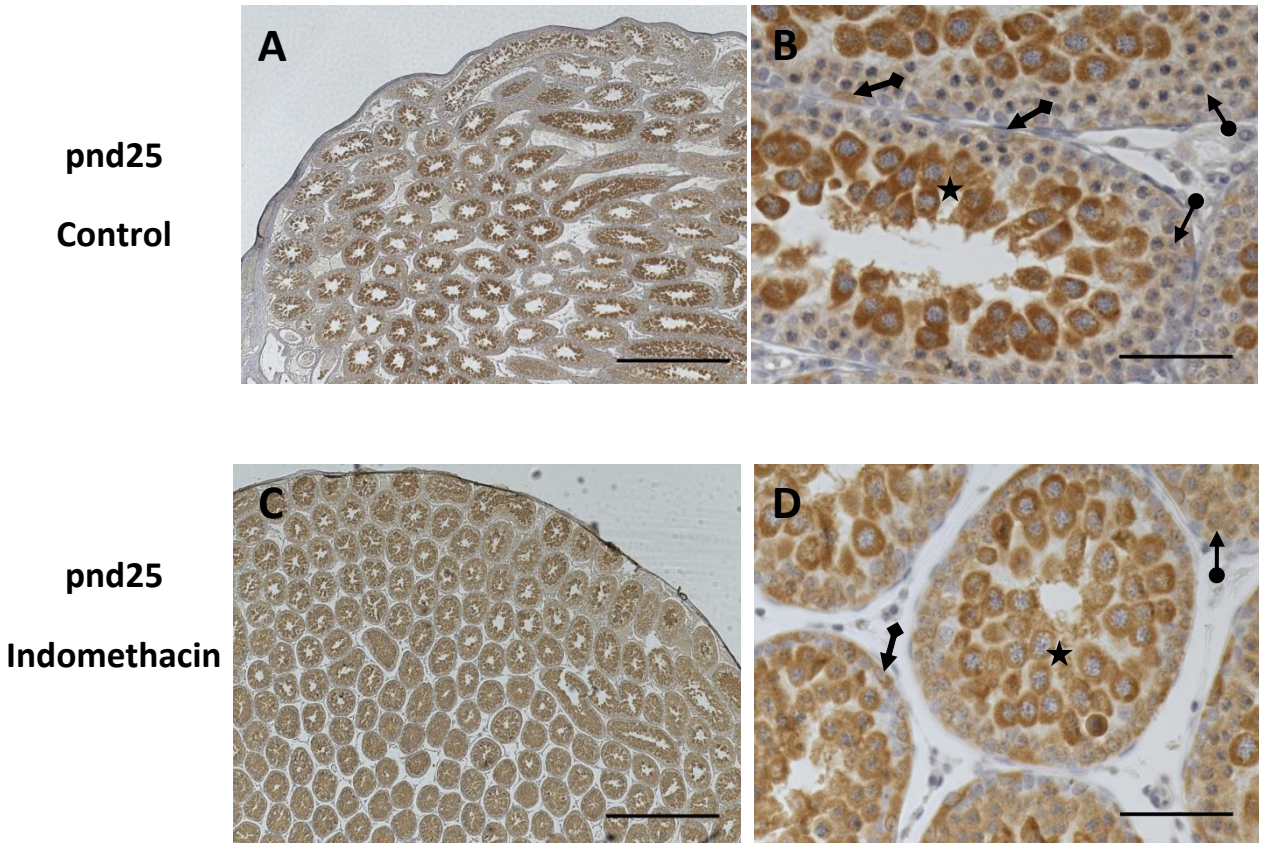


Figure 14. Images of control and indomethacin exposed (1mg/kg or 0.8mg/kg from e15.5 – e18.5) rat pnd25 testis sections. Germ cells were immunostained for VASA and stereology was used to assess the number (millions) of spermatogonia (square labelled arrow), early spermatocytes (circle labelled arrow), pachytene spermatocytes (star) and total germ cell number (millions) in control samples (A, B) and indomethacin exposed samples (C, D). Scale bar (A, C) = 500µm; (B, D) = 50µm. pnd, postnatal day.

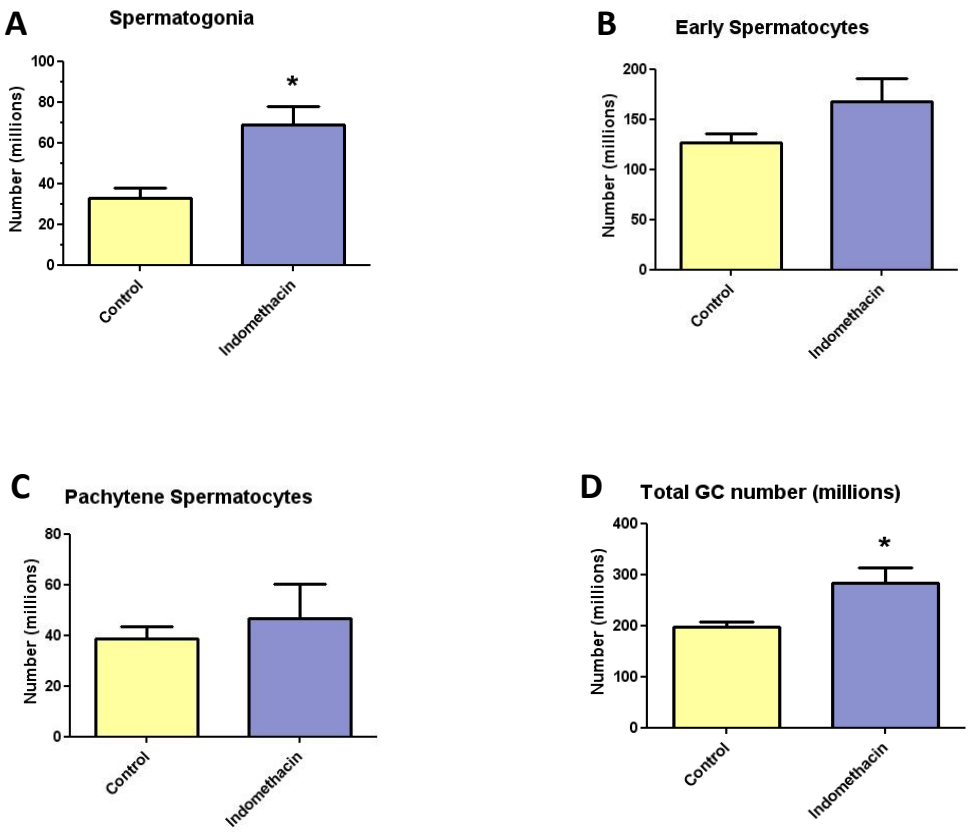


Figure 15. Effect of prostaglandin-inhibitor indomethacin (1mg/kg or 0.8mg/kg from e15.5 – e18.5) on germ cell number of rat pnd25 testis after termination of the treatment. Germ cells were separated in three classes that follow the temporal progression of spermatogenesis: spermatogonia, early spermatocytes and pachytene spermatocytes. The total germ cell number for a sample was also assessed by adding the values of all classes for that sample. Values for control (*n* = 4) and indomethacin (*n* = 4) exposed pnd25 testis were compared using a Student's unpaired *t*-test (A, D). GC, germ cell. *p<0.05, in comparison with respective control value.

Discussion

DBP effects on male human fetal germ cell differentiation and proliferation

This study focused on the impact that environmental chemical exposure (DBP and MBP) has on human germ cell development during fetal life. It was performed to assess whether DBP exposure affects male human fetal germ cell differentiation and proliferation in a xenograft model (small pieces of human testis xenografted under the back skin of castrated adult male nude mice, to which the treatments were given). The results show that the percentage of OCT3/4^{+} (undifferentiated) germ cells is lower in human fetal xenografts exposed to DBP (or its active metabolite MBP). Correspondingly, the percentage of MAGE-A4^{+} (differentiated) germ cells was increased in DBP-exposed xenografts. Nevertheless, both types of germ cells maintained control levels of germ cell proliferation in DBP-exposed samples. Germ cells switch the expression of OCT3/4 to MAGE-A4 when they mature (differentiate), which coincides with reduction/loss of proliferative activity, reassuring that the xenograft model is recapitulating the normal development of male human fetal germ cells.

The reduction in undifferentiated germ cells in DBP-exposed xenografts could be explained either by selective death of these cells or an increase in their differentiation. To distinguish between these hypotheses, one would have to count all germ cells in the testis, which is impossible since the model uses fetal testis xenografts which represent only small component parts of the overall fetal testis. From analysis of the photographs of the human xenografts, selective death of the undifferentiated germ cells seems to be more likely. If indiscriminate apoptosis of germ cells had occurred, the proportion of undifferentiated (OCT3/4^{+}) and differentiated (MAGE-A4^{+}) germ cells would have remained the same for control and DBP-exposed male human fetal testis xenografts. However, my results show that these proportions changed after exposure to DBP, with a relative increase in the proportion of differentiated germ cells. Therefore, it is plausible that there could have been selective apoptosis of undifferentiated germ cells. This would agree with previous reports in the mouse, in which *in vitro* exposure of fetal testis to phthalates induced apoptosis of the germ cell lineage during fetal and postnatal life (Lehraiki *et al.*, 2009). Another study in the rat showed that *in utero* exposure to DBP differentially reduced the numbers of OCT3/4^{+} fetal germ cells (Jobling *et al.*, 2011). Moreover, Lambrot *et al.* (2009) also obtained a similar result for *in vitro* cultures of human fetal testis pieces, where phthalate exposure induced a higher rate of germ cell apoptosis, thus reducing the germ cell number, with no alteration of proliferation. Additionally, Muczynski *et al.* (2012)

recently reported similar results for *in vitro* exposure of human fetal testis cultures and *in utero* exposure of mouse fetal testis to MEHP (the monoester metabolite that results from the metabolization of DEHP, the most widely used phthalate), both resulting in an increased apoptosis of developing germ cells. In all of these in vitro studies, the authors did not investigate if the increased apoptosis applied more to undifferentiated rather than to differentiated germ cells.

Hrabalkova (2011) conducted preliminary studies on human fetal testis xenografts from DBP-exposed (or its active metabolite MBP) nude mice and also reported a difference in proportions of undifferentiated and differentiated germ cells between control and DBP exposed xenografts, which the present studies confirm. The xenografting technique used in both studies was developed by Mitchell *et al.* (2010) and the xenografts show normal structure, function and development, which represent a comparable *in vivo ex situ* model of normal germ cell development. The findings that this model provided could be important for future assessments of exposure to environmental chemicals that affect the development of the human male gonad, since the results are novel.

Indomethacin effects on male rat fetal germ cell number

This study also focused on how *in utero* exposure to paracetamol and indomethacin affects male germ cell number during fetal and postnatal life in rats. This was performed by analysing rat e21.5 and pnd25 testes, which showed the effects of paracetamol/indomethacin exposure during the masculinization programming window in the rat. These medicines are nonsteroidal anti-inflammatory drugs that inhibit prostaglandin production or action. Hrabalkova (2011) reported in preliminary studies that fetal exposure of rats to paracetamol resulted in a decreased number of germ cells at the later stage of fetal development (e21.5). Indomethacin was used to further evaluate this finding, since it is a more unequivocal prostaglandin inhibitor, and, indeed, e21.5 fetal testes from indomethacin-exposed pregnant rats showed a reduction in germ cell number when compared to control fetal testes. However, fetal testes from rats exposed to paracetamol only showed a small trend towards a decreased number of germ cell, without reaching significance. This might be explained by the different methods used to count germ cells compared with Hrabalkova (2011), as stereology provides a more accurate and unbiased means of quantifying the cellular composition of the whole testis. It is still unknown if the germ cell number decrease in

rat e21.5 testes after indomethacin exposure is due to apoptosis. However, an increase of the differentiation rate of fetal germ cells exposed to indomethacin, therefore stopping proliferation at an abnormally early age, could be suggested. Nevertheless, a recent *in vitro* study on human fetal oocytes reported increased apoptosis of organ cultures exposed to dexamethasone, a synthetic glucocorticoid and a known inflammation modulator (Poulain *et al.*, 2012). Duffy *et al.* (2010) reported that monkey and mouse adult oocytes express functional prostaglandin receptors. A COX-2 inhibitor has been shown to directly influence oocyte nuclear maturation and reduced the *in vitro* fertilization rate, blocking several steps in female reproduction (Duffy & VandeVoot, 2011). Unpublished preliminary results by Afshan Dean have shown that COX-2 and prostaglandin receptors expression could also be found in male fetal germ cells in the rat. A recent paper also reported that juvenile mouse Sertoli cell lines exposed in vitro to environmental chemicals showed prostaglandin synthesis inhibition (Kristensen *et al.*, 2011b). Moreover, administration of COX inhibitors results in testicular damage by inducing germ cell apoptosis in postnatal mice (Kubota *et al.*, 2011). Although information about the importance of prostaglandins during testis development is still scarce, these findings altogether suggest that the fetal germ cell line, at least in mammals, needs prostaglandins to assure its normal development and maintain fertility.

On the other hand, this study also analysed the effect of *in utero* indomethacin exposure on germ cell numbers of rat pnd25 testis. This is novel as studying rat pnd25 testis helps to understand if *in utero* exposure to a prostaglandin-inhibitor may cause permanent consequences, which has not been addressed yet. Because spermatogenesis is occurring during this postnatal stage, germ cells were separated into categories that follow the temporal progression of germ cell development into spermatozoa: spermatogonia, early spermatocytes and pachytene spermatocytes. The total germ cell number was also compared between pnd25 testes of vehicle-exposed controls and those exposed in utero to indomethacin. Spermatogonial and total germ cell numbers showed a significant increase in indomethacin exposed animals, when compared to control samples. However, the number of early and pachytene spermatocytes only show an upward trend in pnd25 exposed to indomethacin. This indicates that there might be slow germ cell replenishment once the exposure to indomethacin has stopped (e18.5; 4 days before birth). In adulthood, due to that replenishment, germ cell numbers are expected to be normal. The fact that only one germ cell type (the more undifferentiated type) shows a significant increase in numbers after exposure to indomethacin might be related to its proliferative features, since there might be apoptosis of more differentiated germ cells that do not proliferate and replenish as quickly as spermatogonia. Therefore, *in utero* exposure do indomethacin seems to trigger apoptosis and a staggered recovery of germ cell numbers after birth, as the surviving germ cells (i.e. those that resisted apoptosis) could recover and trigger compensation to achieve normal germ cell numbers.

To conclude, the results were successful in showing that fetal exposure to common environmental chemicals, such as phthalates and the prostaglandin-inhibitor indomethacin, can affect fetal germ cell development in human male and the rat, addressing this issue for the first time. Ongoing studies are focused on analysing the effects of the studied environmental chemicals in females. It is still unclear what effect this might have, but if the results mirror those described in this study, exposure to environmental chemicals becomes even more problematic. Germ cell number in females is fixed prior to birth, so any treatment-induced loss of germ cells that occurs at this time cannot be compensated for after birth, unlike in males, and thus could be translated into serious fertility lifespan issues.

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Appendix

Table I. Germ cell numbers (millions) for e21.5 rat testis exposed to vehicle (control), paracetamol or indomethacin treatment, which were used in statistical analysis.

Treatment		
Control	**Paracetamol**	**Indomethacin**
0,088592	0,20304	0,160091
0,265907	0,125893	0,146815
0,156085	0,14649	0,120480
0,145142	0,125964	0,134816
0,176376	0,259754	0,108308
0,257688	0,229299	0,162236
0,298938	0,240926	0,162923
0,358289	0,28822	0,257175
0,292778	0,295521	0,141253
0,362374	0,252303	0,136963
0,373113		0,183987
0,325335		0,095837
		0,118078
		0,112955

Table II. Spermatogonia, early spermatocytes, pachytene spermatocyes and total germ cell numbers (millions) for pnd25 rat testis exposed to vehicle (control) and indomethacin treatment. These numbers were then used for statistical analysis.

Spermatogonia

Treatment	
Control	**Indomethacin**
29,10129	47,49326
36,20188	61,28696
21,29265	87,92276
44,965245	77,93825

Early Spermatocytes

Treatment	
Control	**Indomethacin**
105,846	189,9936
115,3677	164,1512
134,8296	106,3772
148,94257	210,2121

Pachytene Spermatocytes

Treatment	
Control	**Indomethacin**
38,94679	28,0884
49,8942	29,23583
39,08737	44,26174
26,020922	85,46898

Total Germ Cells

Treatment	
Control	**Indomethacin**
173,8941	265,5753
201,4638	254,674
195,2096	238,5617
219,93	373,6194